

Food allergens are transferred intact across the rat blood-placental barrier in vivo

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Abstract We investigated the mechanism of transplacental macromolecular transport in rats on the nineteenth day of pregnancy using tracers, transmission electron microscopy and immunohistochemistry. The blood-placental barrier of full-term rat placentas was composed of a trilaminar layer of trophoblast cells that separates the fetal capillaries from the maternal blood spaces: a layer of cytotrophoblasts lining the maternal blood space and a bilayer of syncytiotrophoblast surrounding the fetal capillaries. Horseradish peroxidase, intravenously injected into the maternal circulation, was found in the maternal blood spaces, the interspaces between the cytotrophoblasts and the syncytiotrophoblast I, many pits and small vesicles in the syncytiotrophoblast I, vesicles of the syncytiotrophoblast II, fetal connective tissue and fetal capillaries. Intravenously injected ovalbumin was detected in the maternal blood spaces, a trilaminar layer and the fetal capillaries. Neonatal Fc receptor (FcRn), a receptor for IgG, was localized at the maternal side of the blood-placental barrier. These results show that the structure of the rat blood-placental barrier is quite similar to the human blood-placental barrier, and non-specific macromolecules and

food allergens may penetrate through the blood-placental barrier of the full-term placenta from the maternal to fetal circulation mediated by FcRn.

Keywords Placenta · Blood-placental barrier · Transcytosis · Neonatal Fc receptor (FcRn) · Immunoglobulin G (IgG) · Ovalbumin

Introduction

The placenta is an organ that develops during pregnancy in the lining of the uterus. When fully developed, it has the shape of a flat cake. Its primary function is to permit substances in the blood of the mother to diffuse to the blood of the fetus. Under normal conditions, the blood of the mother and the fetus do not mix or come into direct contact with each other, as they are separated by the blood-placental barrier. In the placenta, nutrients and oxygen dissolved in the mother's blood diffuse through the barrier into the blood stream of the fetus, thereby supporting life and growth of the fetus until it is born.

An increasing number of studies show an inconsistent relationship between maternal allergen exposure and either the cord blood response to allergens or the subsequent development of disease [1–4]. Maternal-to-fetal allergen transport occurs in a dose-dependent and molecular weight-dependent manner, particularly in preterm placentas [5]. Nevertheless, this knowledge does not preclude further detailed study on possible routes of exposure of the fetus to allergens. Two possible routes of intrauterine allergen transfer have been postulated: passage of maternally derived allergens from the maternal vasculature of the decidua across the amniochorionic membranes into the amniotic fluid, and passage across the placenta from the

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maternal to the fetal circulation [4]. Transplacental transfer of non-specific macromolecules including allergens has been shown in the choriocarcinoma cell line BeWo [6] and in ex vivo placental models [6–8] at light microscopic level. Bisphenol A is a well-known endocrine disruptor. Its transfer from the maternal blood vessels to the fetus via the placenta has been confirmed by liquid chromatography/time-of-flight mass spectrometry and immunohistochemical analysis [9]. However, the possible routes of intra-uterine allergen transfer in vivo have not yet been well defined.

The neonatal Fc receptor for IgG (FcRn) has been well characterized in the transfer of passive humoral immunity from mother to fetus. Therefore, FcRn expressed on the placenta, intestinal epithelia, liver, lung, and vascular endothelium plays an important role in the transport of maternal IgG [10]. In adults, FcRn regulates the persistence of both IgG and albumin in the serum, as well as the movement of IgG and any bound cargo between different compartments of the body across polarized cells via transcytosis [11]. In humans, IgG transfer across the syncytiotrophoblast of chorionic villi is mediated by FcRn [12–14]. Recent studies have indicated that the transfer of maternal IgG regulates the development of allergic airway inflammation early in life in an FcRn-dependent manner [15].

In this study, we investigated whether non-specific macromolecules and food allergens are transported through the blood-placental barrier from the maternal blood to the fetal blood stream, using tracers and transmission electron microscopy, and we studied the localization of FcRn with immunofluorescence microscopy.

Materials and methods

Animals

Adult female Wistar rats were mated with males of the same strain. The day of sperm detection in vaginal smears was designated as day 0 of gestation. The rats, weighing 374.1 ± 19.3 g, were used on the nineteenth day of pregnancy. The protocol for the research project was approved by a suitably constituted ethics committee of Nakamura Gakuen University and it conformed to the provisions of the Declaration of Helsinki, 1995 (as revised in Edinburgh, 2000).

Light microscopy

The pregnant rats were anesthetized with ether, and the lower abdominal cavity was opened. The fetus and the umbilical cord were exposed after the uterine wall and fetal

membranes had been carefully incised far enough from the attachment site of the placenta. The placentas were detached from the fetus immediately. For light microscopic study, the specimens were fixed in 4.0 % paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4) for 2 h at 4 °C, washed in 0.1 M PB and dehydrated in a graded series of ethanol before being replaced in xylene, and embedded in paraffin wax. Thin sections were cut into 6- μ m slices using a microtome, were stained with hematoxylin and eosin, and were observed under a light microscope.

Transmission electron microscopy

For the transmission electron microscopic study, tissues were cut into 1.0 $\times$ 2.0 mm blocks, fixed in a mixture of 2.0 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M PB (pH 7.4) for 2 h at 4 °C. Aldehyde-fixed tissue samples were postfixed for 2 h at 4 °C in an aqueous solution of 1.0 % osmium tetroxide. After dehydration in a graded series of ethanol, they were embedded in EPON 812. Toluidine blue stained 1- μ m sections were observed under a light microscope. For the ultrastructural study, ultrathin sections were stained with uranyl acetate and lead acetate, and were examined under a transmission electron microscope (JEM-1200EX, JEOL, Tokyo, Japan) accelerating at 80 kV.

Macromolecular tracer uptake study

As a non-specific macromolecular tracer, horseradish peroxidase (HRP: Type II, MW 44 kDa, Sigma-Aldrich, St. Louis, MO, USA) was injected into the maternal inferior vena cava (350 mg/kg). After 1 h, the placentas were collected immediately and immersed into fixative. To detect the localization of HRP, tissues were cut into 1.0- to 2.0-mm blocks, fixed for 2 h at 4 °C in a mixture of 2.0 % paraformaldehyde and 2.5 % glutaraldehyde in 0.1 M PB (pH 7.4). Specimens were washed overnight in 0.1 M PB at 4 °C. Tissues were treated for 1 h at room temperature with 0.05 % 3,3'-diaminobenzidine (DAB) and 0.01 % hydrogen peroxide in 0.1 M cacodylate buffer (pH 7.4). All specimens were rinsed in the 0.1 M PB, postfixed for 2 h at 4 °C in an aqueous solution of 1.0 % osmium tetroxide, dehydrated and embedded in EPON 812. Ultrathin sections stained with lead acetate were examined using a transmission electron microscope accelerating at 80 kV.

Immunohistochemistry

As a well-known food allergen, ovalbumin (albumin from chicken egg white, Sigma-Aldrich, St. Louis, MO, USA) was injected into the lumen (350 mg/kg) of the mother's

inferior vena cava. As a control, physiological saline was injected. After 1 h, the placentas were removed intact, immersed in fixative in 4.0 % paraformaldehyde in 0.1 M PB (pH 7.4) for 2 h at 4 °C. For the light microscopic study, the samples were embedded in paraffin wax and cut into 6- μ m slices.

For detection of ovalbumin or FcRn, deparaffinized sections were treated with microwave at 800 W (MICROMED T/T; Milestone, Sorisole, Italy) for 20 min. Antigen-retrieved sections were blocked with 1.0 % bovine serum albumin (BSA)–phosphate buffered saline (PBS) for 1 h at room temperature. To detect ovalbumin or FcRn localization, the primary antibody used was rabbit anti-chicken ovalbumin antibody (Bethyl Laboratories, Montgomery, TX, USA) or rabbit anti-FcRn antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were incubated with the primary antibody diluted 1:100 in 1.0 % BSA–PBS for 2 h at room temperature. For detection of FcRn, the sections used were from untreated rats. As negative control, sections were treated with only 1.0 % BSA–PBS. As positive control for detection of FcRn, the sections of suckling rat jejunum were used. After being washed three times with PBS, all the sections were incubated with secondary antibody diluted 1:500 in 1.0 % BSA–PBS for 1 h at room temperature. As secondary antibody, Alexa Fluor 488-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR, USA) was used. The sections were washed three times with PBS, mounted with an Ultra Cruz Mounting Medium (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and observed with a fluorescence microscope (Axio Imager. A1, Carl Zeiss, Göttingen, Germany).

Results

Structure of the full-term placenta

We first analyzed the structure of rat placentas on day 19 of pregnancy using light and transmission electron microscopy. The placenta was composed of tissues of both maternal and fetal origin. The labyrinth zone, basal zone, yolk sac, amnion and allantoic membrane were structural components of fetal origin. The decidua basalis and metrial glands were structural components of maternal origin (Fig. 1). In the labyrinth, there were maternal and fetal blood spaces. A higher magnification view of the labyrinth showed that maternal blood spaces were much larger and were irregularly shaped. Fetal red blood cells were different from maternal ones in that they were much larger in size (Fig. 2, inset). The placental membrane was observed in the blood-placental barrier of the labyrinth. The placental membrane was visualized by transmission electron

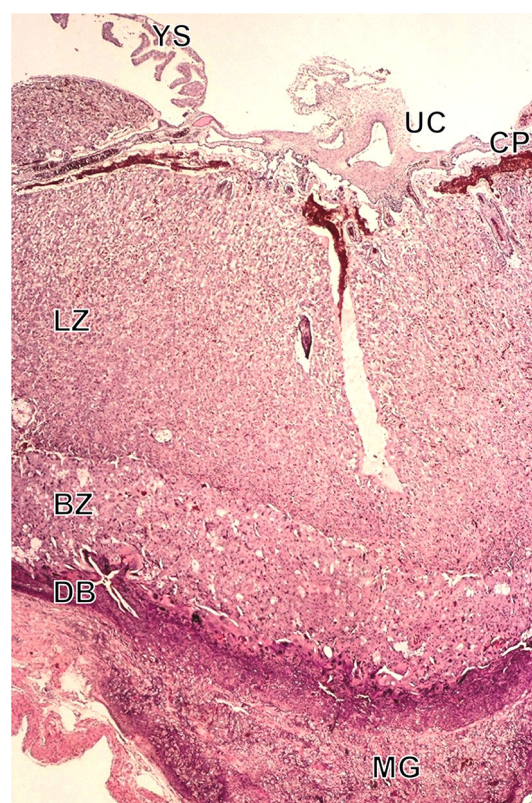


Fig. 1 Light micrograph of a full-term rat placenta. The umbilical cord (UC), chorionic plate (CP), labyrinth zone (LZ), basal zone (BZ) and decidua basalis (DB) can be observed. YS yolk sac, MG metrial gland

microscopy (Fig. 2). The first layer cytotrophoblast, which directly faces the maternal blood space, was a flat, porous membrane. The cytotrophoblast had large nuclei, rough endoplasmic reticulum, free ribosomes, mitochondria and lysosomes. The second layer was syncytiotrophoblast I. The syncytiotrophoblast I had extensive surface folding on the cytotrophoblast side. Desmosomes could be observed between the cytotrophoblasts and the syncytiotrophoblast I. The third layer, syncytiotrophoblast II, was located under the syncytiotrophoblast I. The membranes of the syncytiotrophoblast I and II were closely apposed to each other and had a scalloped appearance. Between the syncytiotrophoblast I and II, a lot of gap junctions could be observed. In the syncytiotrophoblasts I and II, there were smooth and rough endoplasmic reticulum that had dilated lumens, free ribosomes, mitochondria and lysosomes. In addition, vesicles could be observed in these cells. The number and size of the vesicles were different between syncytiotrophoblast I and II. A lot of small vesicles were observed in the syncytiotrophoblast I, and vesicles were larger and the number of vesicles was lower in the syncytiotrophoblast II than in the syncytiotrophoblast I. On the basal side of the syncytiotrophoblast II, there was a complex system of

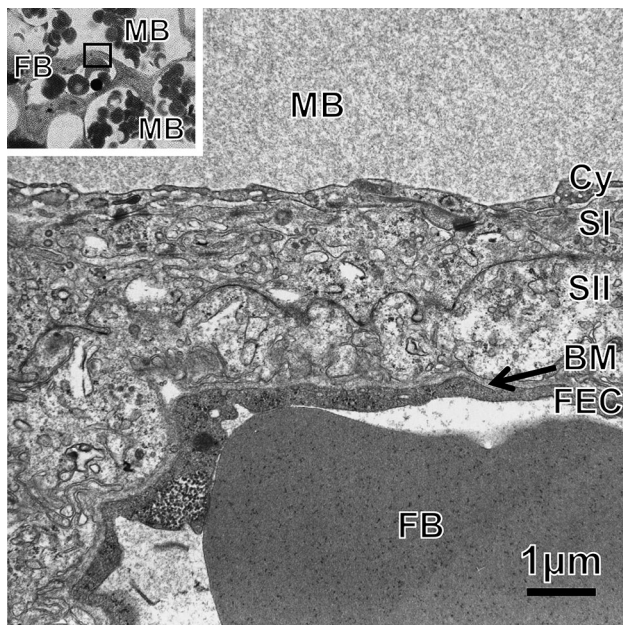


Fig. 2 Transmission electron micrograph of the blood-placental barrier shown in the *boxed area* of the *inset*. The *inset* is a higher magnification light micrograph of the labyrinth. Fetal red blood cells are different from those of the mother in that they are larger in size. Three trophoblast layers [cytotrophoblast (Cy), syncytiotrophoblast I (SI) and syncytiotrophoblast II (SII)] and fetal endothelial cells (FEC) separate the maternal blood space (MB) from the fetal blood space (FB). Red blood cells are observed in the fetal blood space. BM basement membrane. Bar 1 μ m

infoldings that reached deep into the cytoplasm. Under the syncytiotrophoblast II, the basement membrane was placed between the fenestrated endothelial cells of the fetal capillaries. The fetal endothelial cells had electron-dense cytoplasm with rough endoplasmic reticulum, a lot of free ribosomes, mitochondria, and lysosomes.

Permeability of macromolecules in the placenta

We next determined the permeability of HRP, a macromolecular tracer, across the blood-placental barrier using transmission electron microscopy. In the lower magnification, HRP injected into the maternal circulation could be observed in the maternal blood spaces and between the pores of cytotrophoblasts (Fig. 3a). A lot of small pits, small vesicles and lysosomes filled with tracer were observed in the syncytiotrophoblast I. The tracer was rarely observed in the syncytiotrophoblast II at low magnification.

In the lumen of the fetal capillaries, reaction products of HRP could be identified (Fig. 3a). Higher magnification views showed the details of tracer distribution (Fig. 3b). Reaction products could often be observed through the pores of the cytotrophoblasts. In the syncytiotrophoblast I, there were a lot of coated pits and small vesicles containing HRP, especially on the cytotrophoblast side. Reaction

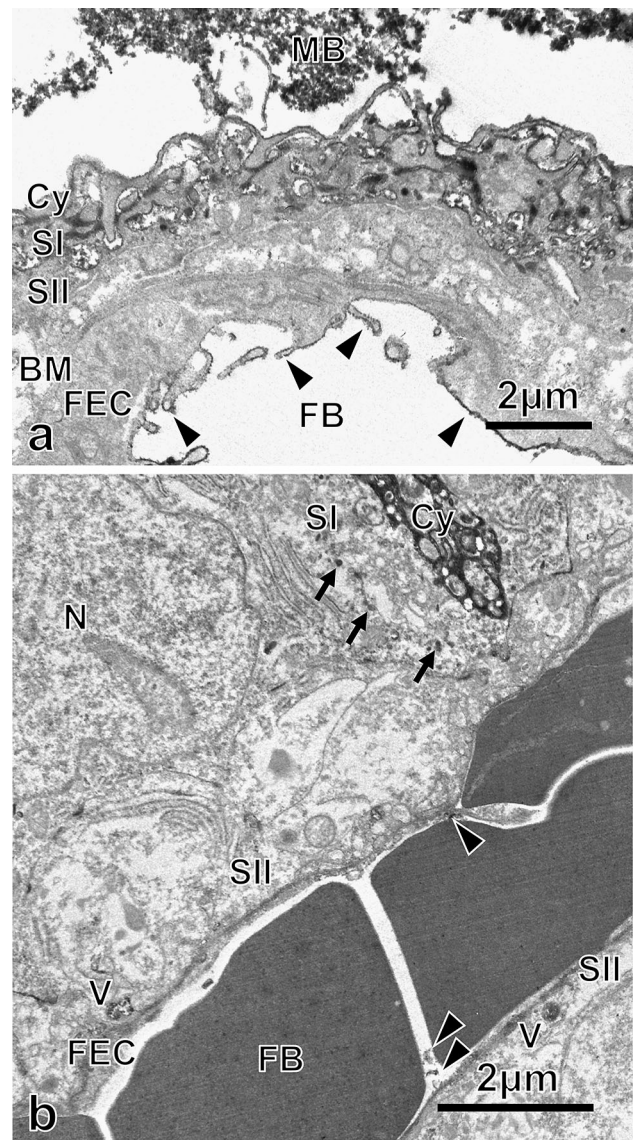


Fig. 3 Transmission electron micrographs of the blood-placental barrier 1 h after intravenous injection of HRP. **a** The lower magnification view shows the presence of HRP in the maternal blood space (MB), cytotrophoblasts (Cy), syncytiotrophoblast I (SI), syncytiotrophoblast II (SII), fetal endothelial cell (FEC) and inside (arrowheads) the fetal blood space (FB). **b** Higher magnification view of the blood-placental barrier. Reaction products can be seen between the pores of the cytotrophoblast, in many of the small vesicles (arrows) of the syncytiotrophoblast I, the intercellular space, pits and large vesicles (V) of the syncytiotrophoblast II, interstitium, between the fenestra of the fetal endothelial cells and inside (arrowheads) the fetal blood space. N nucleus. Bars 2 μ m

products were seen in the interstice between the syncytiotrophoblast I and II. A few large vesicles filled with tracer were observed in the syncytiotrophoblast II. Moreover, the tracer was observed within the intercellular space between the syncytiotrophoblast II and the endothelial cells of the fetal capillaries. HRP could also be detected in the fenestrae of fetal endothelial cells and in the fetal capillary (Fig. 3b).

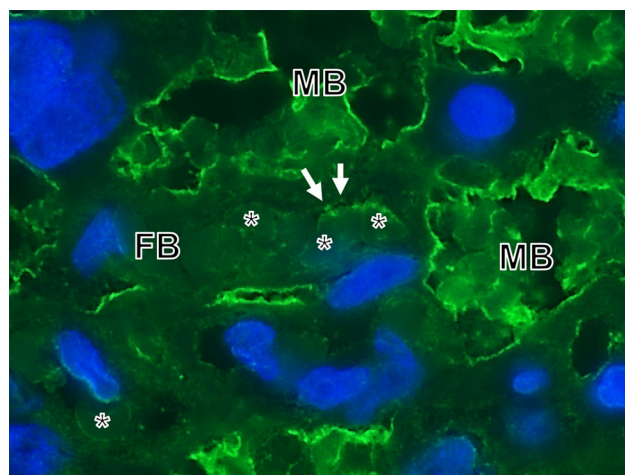


Fig. 4 Localization of intravenously injected ovalbumin in the labyrinth. Ovalbumin antibody is detected (*green signal*) not only in the maternal blood space (*MB*) but in the fetal blood space (*FB*, *arrows*). Nuclei are stained with DAPI (*blue*). Asterisks indicate fetal red blood cells

Penetration of ovalbumin into the fetal circulation

We next injected ovalbumin, a typical food allergen, into the maternal circulation, and analyzed its distribution across the blood-placental barrier using immunohistochemistry. Intravenously injected ovalbumin could be observed in the maternal blood vessels, the placental membranes of the labyrinth and in the fetal capillaries (Fig. 4).

Localization of FcRn in the blood-placental barrier

Finally, to elucidate whether FcRn mediates macromolecular penetration, we examined the localization of FcRn in the full-term placenta using immunohistochemistry. Since FcRn is a receptor for IgG that was first found in suckling rodents, we used sections of suckling rat jejunum as a positive control. FcRn was widely distributed in the apical cytoplasm of the jejunal absorptive cells of suckling rats (Fig. 5a). In the placenta, FcRn was widely distributed in the cytoplasm of the cells of the yolk sac (Fig. 5b). In the placental membranes of the labyrinth, FcRn was localized in the maternal side of the bilayer of the blood-placental barrier (Fig. 5c).

Discussion

We investigated the mechanism of transplacental macromolecular transport in rats on day 19 of pregnancy using tracers, transmission electron microscopy and immunohistochemistry. We showed the fine structure of the blood-placental barrier and revealed the transplacental route

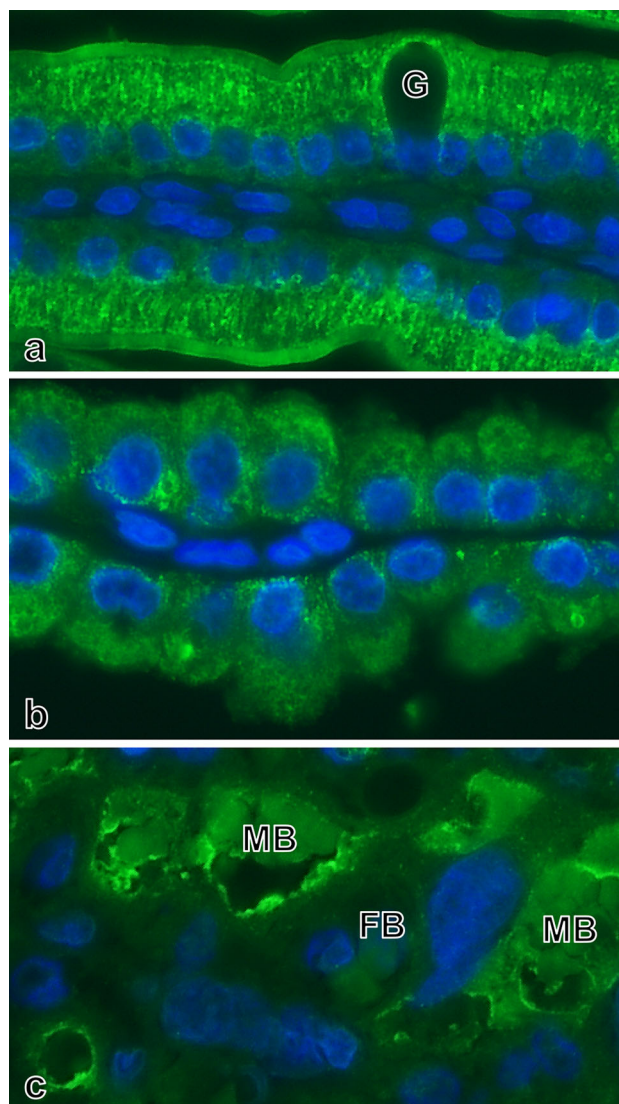


Fig. 5 Localization of FcRn. **a** Jejunal absorptive cells of a suckling rat. **b** Yolk sac cells of the full-term placenta. **c** Labyrinth of the full-term placenta. FcRn antibody can be detected (*green signal*) in the jejunal absorptive cells, yolk sac cells and on the maternal side of the bilayer of the blood-placental barrier. Nuclei are stained with DAPI (*blue*). *G* goblet cell, *MB* maternal blood space, *FB* fetal blood space

of macromolecules, including a food allergen, in the fetal–maternal interface from the maternal to the fetal circulation. Finally, we showed the possibility of partial participation of FcRn in macromolecular translocation across the placenta.

Although the gross architecture of human and mouse placentas differs somewhat in detail, their overall structure and the molecular mechanisms underlying placental development are thought to be quite similar. As a result, mice are increasingly used as a valuable animal model for studying the many aspects of placentation [16–19]. However, in contrast with the human placenta, the intrauterine trophoblast invasion in mice is shallow and placentation is

superficial [20–22]. In the rat, the organization of placental sites exhibits striking similarities with that of the human. Both rat and human placentas exhibit deep trophoblast invasion [23], although the cellular arrangement of placental barrier differs between rat and human [24]. As rats have many advantages over mice, including the organization of body, physiology, and capacity for genetic manipulation [25, 26], the rat has been the preferred animal model for physiologists. Furthermore, its size makes it more amenable to experimental manipulation, resulting in considerable understanding of rat physiology and its similarities with human [24].

Previous studies [27–31] and our ultrastructural study showed that the fully developed rat placenta is composed of three major layers. We referred the porous cell layer lining the maternal blood spaces, cytotrophoblast, the maternal side of bilayer of syncytiotrophoblast, syncytiotrophoblast I, and fetal side, syncytiotrophoblast II. Recent studies have classified cytotrophoblasts into four subtypes based on time, location, and function of their secretory hormones and paracrine factors in the mature mouse placenta [31]. The difference between syncytiotrophoblast I and II, however, remains unclear. In our observations, the membrane system and the macromolecular transport system varied in these two types of syncytiotrophoblast cells.

Previous studies using HRP and/or lanthanum chloride as tracers in the chorioallantoic rat placenta reported that the main barrier preventing the permeation of macromolecules from the maternal circulation to the fetus is the syncytiotrophoblast I [28] and the barrier preventing permeation from the fetal to the maternal circulation is the syncytiotrophoblast II [29]. In the case of polystyrene particles, the syncytiotrophoblast II is thought to be the barrier in the full-term rat placenta [30]. In our tracer study, differences in the transcytosis patterns of macromolecules were apparent between the syncytiotrophoblast I and II. HRP was observed in the abundant coated pits and small vesicles of the syncytiotrophoblast I. In contrast, endocytosed tracer was only found in a few pits and a few larger vesicles in the syncytiotrophoblast II. This suggests that receptor-mediated endocytosis may occur in the syncytiotrophoblast I, while fluid-phase endocytosis may occur in the syncytiotrophoblast II.

In the human placenta, two cellular barriers regulate IgG transport: the syncytiotrophoblast and the fetal capillary endothelium [32, 33]. However, the fetal capillary endothelium of rats was fenestrated. FcRn, a receptor for IgG [34] is located in the syncytiotrophoblast of the human placenta and transports maternal IgG across the first barrier [10, 12, 13]. FcRn was localized in the maternal side of the bilayer of the blood-placental barrier, the visceral yolk sac, and the jejunal absorptive cells of suckling rats used as a positive control in our study. Visceral yolk sac cells have a

highly specialized apical membrane system for endocytosis [35]. In mice [36] and transgenic rabbits that overexpress FcRn [37], however, FcRn is expressed in the cells of the yolk sac but not in other cells of the yolk sac placenta or in the chorioallantoic placenta. The expression and localization pattern of FcRn may vary in species. Differences in the distribution pattern of FcRn between the yolk sac and placental membrane may be due to the cellular shape. The localization of FcRn may associate with the difference in endocytosis between syncytiotrophoblast I and II.

Two possible routes have been postulated for intrauterine allergen transfer: passage of maternally derived allergens from the maternal vasculature of the decidua across the amniochorionic membranes into the amniotic fluid, and transfer across the placenta from the maternal to the fetal circulation [4]. In this study, we tried to elucidate the latter route. In the immunohistochemical analysis, intravenously injected ovalbumin was detected in the maternal blood spaces, the blood-placental barrier and the fetal capillaries, similar to HRP. These results support the possibility that non-specific macromolecules of at least 40 kDa can penetrate from the maternal blood. As transcytosis does not occur in the jejunal absorptive cells from neonatal rats before suckling [38], we suggest that the transfer of macromolecules across the amniochorionic membrane is low.

Our study provides evidence for the intrauterine allergen transfer route across the placenta from the maternal to the fetal circulation and the participation of FcRn.

Conclusion

Our study showed the fine structure of the blood-placental barrier, especially in difference between syncytiotrophoblast I and II, and revealed the second possible route of macromolecular transport including food allergen across the blood-placental barrier in vivo using tracer and transmission electron microscopy. It was suggested that FcRn-associated IgG transport was partially participated in the passage of maternally derived allergen from the maternal vasculature.

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